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# EGFR protein overexpression and gene copy number increases in oral tongue squamous cell carcinoma

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ABSTRACT

New promising therapeutic agents targeting epidermal growth factor receptor (EGFR) have been developed although clinical information concerning EGFR status in oral tongue squamous cell carcinoma (OTSCC) is limited. We investigated EGFR protein expression and gene copy numbers in 78 pretreatment OTSCC paraffin samples. EGFR protein expression was found in all 78 tumours, of which 72% showed an intense staining. Fifty-four percent of the tumours had high (⩾four gene copies) EGFR gene copy numbers. EGFR gene copy number was significantly associated with EGFR protein expression ($P = 0.002$). Pretreatment EGFR staining intensity tended to be associated with non-pathological complete remission after preoperative radiotherapy for Stage II OTSCC. No correlation was found between EGFR status and survival. EGFR FISH results were significantly ($P = 0.003$) higher in more advanced tumours (Stages II, III and IV) than in the tumours in Stage I. Non-smokers exhibited a significantly higher EGFR gene copy number and protein overexpression in Stages I and II OTSCC than smokers ($P = 0.001$, $P = 0.009$). In conclusion, EGFR was found to be overexpressed in all OTSCCs making this cancer type interesting for exploring new therapeutic agents targeting the EGFR receptor.



## 1. Introduction

Oral tongue squamous cell carcinomas (OTSCCs) are associated with increasing incidence and poor prognosis.[1] Deeper understanding of the molecular behaviour of the tumour may improve patient outcome. New promising therapeutic agents targeting epidermal growth factor receptor (EGFR) have been developed but clinical information concerning EGFR status in OTSCC is limited.

Epidermal growth factor receptor (EGFR) is a tyrosine kinase (TK) receptor known to be associated with radiation resistance and prognosis in head and neck squamous cell carcinoma (HNSCC).[2,3] Overexpression of EGFR is seen in approximately 90% of head and neck cancers making it an interesting target for therapy.[4,5] EGFR inhibitors, one being C225 (cetuximab), have shown promising radiosensitivity enhancement with amplification of radiation-induced apoptosis in tumour specimens.[6] These studies are highly

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supportive that EGFR might provide a radiosensitising route for OTSCC patients.

This study aimed to investigate EGFR status using immunohistochemistry (IHC) and fluorescence *in situ* hybridisation (FISH) in patients with OTSCC. We investigated the association between EGFR IHC and FISH. In addition, we evaluated whether EGFR protein expression and/or high gene copy numbers, ⩾four gene copies, were associated with tumour progression, treatment prediction of preoperative radiotherapy and prognosis in OTSCC.

## 2. Materials and methods

We obtained 78 formalin-fixed, paraffin-embedded biopsy specimens with histopathologically confirmed OTSCC (UICC Stages I–IV) treated at the Department of Oto-Rhino-Laryngology, Head and Neck Surgery, Karolinska University Hospital (Stockholm, Sweden) from January 2000 to December 2004. Clinical information, including age, tumour grade, treatment modality, treatment response and follow-up, was retrieved from medical records (Table 1). Each patient had a minimum follow-up time of at least 36 months after initial cancer diagnosis (survival range: 3–60 months). All histological samples were reviewed and the grade of differentiation was evaluated by an experienced pathologist who was blinded to clinical outcome. Informed consent was obtained from all subjects and/or guardians in this investigation. The present study was carried out with the approval from the Regional Ethics Committee in Stockholm.

In general, treatment for OTSCC was based on the UICC classification stage and patient performance.[7] Prior to treatment plan, tumour stage was determined by means of ultrasound-guided fine needle aspiration cytology, radiology with CT/MRI and palpation under general anaesthesia. All patients were discussed at a multidisciplinary conference. Stage I-diagnosed OTSCC patients were generally treated with local resection alone. Stage II OTSCC patients were treated with preoperative radiotherapy against the tumour and ipsilateral neck region followed by hemiglossectomy. Individual treatment was applied to patients diagnosed with OTSCC Stages III and IV according to tumour size and spread, with surgery and/or radiotherapy and/or chemotherapy. Preoperative radiation therapy was given with a mean total dose of 50–68 Gy, depending on clinical and tumour characteristics.

Two 4-μm haematoxylin-eosin (HTX)-stained sections were cut before and after IHC and FISH sections were obtained, to confirm tumour representativity. All 78 (Stage I, $n = 23$; Stage II, $n = 34$; Stage III, $n = 8$; and Stage IV, $n = 13$) representative formalin-fixed, paraffin-embedded biopsy specimens were available for EGFR IHC staining. Sixty-five (Stage I, $n = 15$; Stage II, $n = 30$; Stage III, $n = 7$; and Stage IV, $n = 13$) representative specimens were available for FISH. Thirteen samples were eliminated from FISH analysis due to the limitation of tissue.

### 2.1. Immunohistochemical staining and analyses

Tissue sections (4 μm thick) were stained with the commercially available monoclonal antibody, the Zymed EGFR, clone 31G7 (Invitrogen: Carlsbad, CA, USA) using the Benchmark XT system. The Benchmark XT (Ventana Medical Systems: Tucson, AZ, USA) automatically standardised, prepared and stained the 4-μm EGFR sections. Breast tissue and cell line A431 served as the negative and positive controls, respectively, for the EGFR staining. Staining reproducibility was verified by a pathologist at a later date by presenting five previously stained EGFR slides for scoring in comparison to original results. All five slides matched previous scores. Thus, with standardised staining/analysis techniques, the results were reproducible.

The specimens were scored according to previous publications[8,9] by assessing the EGFR staining site for membranous intensity regions. All slides were evaluated using a light microscope at 40X magnification in four representative, independent areas by a pathologist, who was blinded to patient data. The tumour samples were scored into: (a) weak staining; (b) moderate staining; and (c) intense staining. The assessments were compared to the negative control (breast tissue), which provided a baseline for staining evaluation. A re-evaluation of each of the stained cross-sections was performed at a later date to test reproducibility by one of the authors and the pathologist. The Altra20 soft imaging system (Olympus Soft Imaging Solutions: Münster, Germany) in cooperation with the CellB software (2.5, Build 1153), was used to capture the stained images for illustration purposes.

### 2.2. Fluorescence in situ hybridisation and analyses

Dual colour FISH was performed on 65 (6 μm thick) samples using a centromere-specific probe for chromosome 4 and a contig of three overlapping bacterial artificial chromosome (BAC) clones that contained the EGFR gene on the 7p12 locus. The centromere-specific probe for chromosome 4 (CEP4) (Abbott Molecular Inc.: Des Plaines, IL, USA) was labelled in Spectrum Aqua. The EGFR contig was labelled by nick-translation with Spectrum Orange-dUTP (Abbott Molecular Inc.: Des Plaines, IL, USA).

Paraffin tissue sections were deparaffinised using xylene (three times for 10 min), rehydrated followed by 0.04% pepsin digestion for 4 h. The slides were then fixed with a formaldehyde solution for 10 min before being dehydrated in an ethanol series and air dried. Slides were then subjected to slide denaturation in a formamide solution before being put in a cold dehydrating ethanol series (70%, 90% and 100%). The probes were denatured for 5 min at 80 °C followed by preannealing at 37 °C for 2 h. Preannealed probes were then added to denatured slides, coverslipped and sealed with rubber cement before being put in a humidified chamber for overnight hybridisation at 37 °C. After hybridisation, slides were washed in 2 × SSC (three times for 3 min) followed by a dehydrating ethanol series (three times for 3 min). The slides were counterstained and embedded with a 4,6-diamidino-2-phenylindole/antifade solution.

Hybridised FISH slides were viewed using the Leica DM-RXA fluorescence microscope (Leica: Wetzlar, Germany) equipped with custom optical filters at 40× objective. The Leica CW 4000 FISH software was used to acquire multifocal images for each filter used. Fifteen to 25 images were taken in areas of optimal cell density with minimal cellular clumps

**Table 1 – Patient and tumour characteristics according to stage.**

| Characteristics | Stage I[a] $n = 23$ | Stage II[a] $n = 34$ | Stage III[a] $n = 8$ | Stage IV[a] $n = 13$ | Total (%) $n = 78$ |
|---|---|---|---|---|---|
| *Age at diagnosis* | | | | | |
| 20–39 years | 3 | 5 | 1 | 1 | 10 (13) |
| 40–59 years | 10 | 16 | 4 | 2 | 32 (41) |
| >60 years | 10 | 13 | 3 | 10 | 36 (46) |
| *Gender* | | | | | |
| Male | 10 | 23 | 5 | 7 | 45 (58) |
| Female | 13 | 11 | 3 | 6 | 33 (42) |
| *Smoking habit* | | | | | |
| Smoking | 12 | 17 | 5 | 6 | 40 (51) |
| Non-smoker | 7 | 13 | 2 | 2 | 24 (31) |
| No information | 4 | 4 | 1 | 5 | 14 (18) |
| *Histopathologic grade*[b] | | | | | |
| Well differentiated | 9 | 5 | 1 | 3 | 18 (23) |
| Moderately differentiated | 12 | 22 | 7 | 7 | 48 (62) |
| Poorly differentiated | 2 | 7 | 0 | 3 | 12 (15) |
| *Preoperative radiation response* | | | | | |
| pCR | 0 | 7 | 1 | 0 | 8 (10) |
| non-pCR | 0 | 21 | 5 | 3 | 29 (37) |
| *Survival* | | | | | |
| Alive | 18 | 17 | 2 | 3 | 40 (51) |
| Dead | 5 | 17 | 6 | 10 | 38 (49) |
| *Resection status* | | | | | |
| R0 | 23 | 28 | 5 | 4 | 60 (77) |
| R1 | 0 | 1 | 0 | 1 | 2 (3) |
| R2 | 0 | 0 | 0 | 0 | 0 (0) |
| No primary surgery | 0 | 5 | 3 | 8 | 16 (21) |
| *Recurrence* | | | | | |
| Locoregional | 10 | 13 | N/A | N/A | N/A |
| Systemic | 0 | 1 | | | |
| No recurrence | 11 | 20 | | | |
| Secondary primary | 2 | 0 | | | |
| *TNM staging*[d] | | | | | |
| T1 | 23 | 0 | 0 | 0 | 23 (29) |
| T2 | 0 | 34 | 2 | 4 | 40 (51) |
| T3 | 0 | 0 | 6 | 0 | 6 (8) |
| T4 | 0 | 0 | 0 | 9 | 9 (12) |
| *EGFR IHC* | | | | | |
| Intense | 19 | 25 | 3 | 9 | 56 (72) |
| Moderate | 4 | 8 | 4 | 2 | 18 (23) |
| Weak | 0 | 1 | 1 | 2 | 4 (5) |
| *EGFR FISH* | | | | | |
| Positive | 3 | 20 | 4 | 8 | 35 (54)[c] |
| Negative | 12 | 10 | 3 | 5 | 30 (46)[c] |

*Abbreviations*: pCR (complete pathological remission); non-pCR (incomplete pathological remission); R0 (no gross residual disease and negative margins of resection); R1 (residual microscopic disease); R2 (residual gross disease); IHC (immunohistochemistry); and FISH (fluorescence *in situ* hybridisation).

a Tumour stage according to UICC.

b Tumour differentiation grade according to the World Health Organisation International Histological Classification of Tumours.

c Total number of patients for EGFR FISH is 65.

d Tumour stage (TNM) according to AJCC.

and overlapping cells. Nuclei that could not be evaluated due to various reasons, including overlaps and insufficient hybridisation, were excluded. Centromere 4 functioned as the control for each case, with nuclei lacking the centromere probe, not being evaluated. Two hundred and fifty cells were counted for each case.

EGFR FISH patterns were classified into four different groups: disomy, trisomy, low-level gains and high-level gains. Disomy consisted of $\leqslant$two gene copies in more than 90% of the cells. Trisomy is described as three gene copies in more than 10% of cells and $\geqslant$four gene copies in less than 15% of cells. Low-level gains consisted of $\geqslant$four gene copies in

⩾15% of cells but less than 30% of cells. High-level gains included ⩾four gene copies in ⩾30% of cells. Tumours classified as disomy and trisomy were considered FISH negative. Tumours showing low-level gains and high-level gains were considered to be FISH positive.

### 2.3. Statistical analysis

SAS version 9.1 (SAS Institute: Cary, NC) was used for all statistical analyses. The Mantel–Haenszel Chi-square test ($\chi^2$ MH) was used to test patient characteristics with EGFR FISH and IHC scores. The Spearman correlation was used to test for correlations between EGFR FISH and IHC scores. The Stata/IC10 software (Stata: College Station, TX, USA) was used to construct the Kaplan–Meier survival curves, using a 60-month cutoff. Survival was calculated from the date of tumour diagnosis until the time of death from any cause, or in patients who remained alive using the Wilcoxon test. All *P*-values were from a two-sided test with a *P*-value < 0.05 considered to indicate statistical significance.

## 3. Results

EGFR protein expression determined by IHC showed that all tumours were positively stained for EGFR (Table 1). EGFR protein expression was categorised as intense in 72% and weak in 5% of patients, which is illustrated in Fig. 1A and B.

FISH analyses were performed on interphase cells from 65 OTSCC patients. Fifty-four percent (35 out of 65) of OTSCCs were FISH positive and had high gene copy numbers, i.e. ⩾four gene copy numbers (Table 1). In the positive group, there were 20 tumours with high-level gains and 15 tumours with low-level gains. In the FISH-negative group, 19 were considered trisomy while 11 were disomy. FISH images of disomy and high-level gains are shown in Fig. 1C and D.

EGFR FISH results were significantly ($P = 0.003$) higher in advanced tumours (Stages II, III and IV) than in the tumours in Stage I.

To evaluate the association between EGFR protein expression levels with gene copy number, IHC results were compared with FISH categories, as shown in Table 2. EGFR protein expression levels were significantly associated ($P = 0.004$) with EGFR FISH categories in all patients. In addition, correlation analyses showed significant correlation between EGFR protein expression levels and EGFR FISH categories [Spearman correlation coefficient of 0.385 ($P = 0.002$)]. Marker distributions of EGFR IHC and FISH are presented in Fig. 2.

### 3.1. EGFR prediction of preoperative radiotherapy response

Of 37 patients treated with preoperative radiotherapy, 8 showed pathological complete response (pCR) while 29 exhibited incomplete pathological remission (non-pCR). Intense EGFR protein expression and high gene copy number tended to be higher in non-pCR tumours, though not significant, as shown in Table 3.

### 3.2. EGFR correlation to smoking habits

To evaluate the association between EGFR protein and gene expression levels in non-smokers and smokers, comparisons were made according to the stage, as shown in Table 4. Significant associations were found in Stages I and II for both IHC ($P = 0.001$) and FISH ($P = 0.009$), with high protein expression and high gene copy number in non-smokers. High EGFR expression was seen in 83% of non-smokers using immunohistochemistry and in 79% of patients when using FISH.

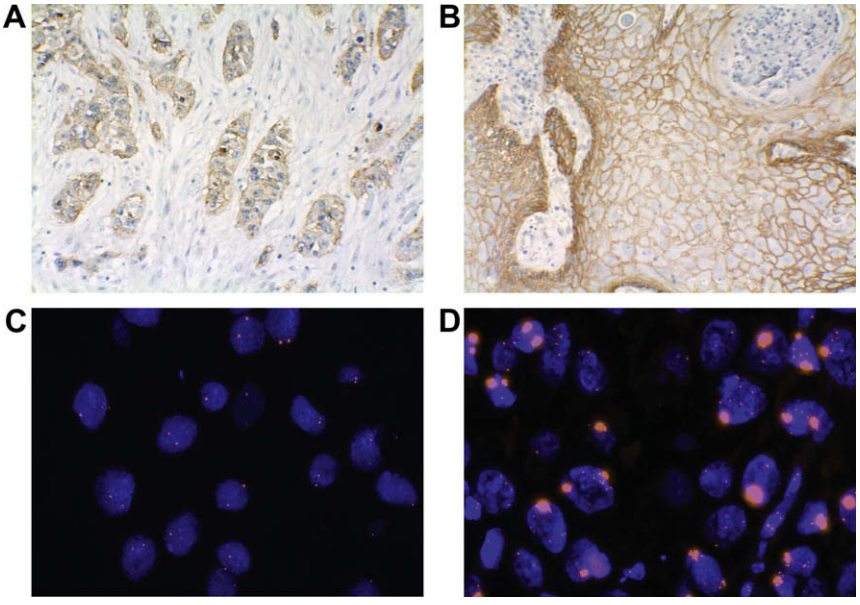


**Fig. 1 – Level of EGFR protein and gene copy number according to immunohistochemistry (IHC) and fluorescence *in situ* hybridisation (FISH) in OTSCC. Panels illustrate specimens graded with: (A) EGFR IHC weak staining, (B) EGFR intense staining, (C) EGFR FISH disomy, and (D) EGFR FISH high-level gains, consistent with homogenously staining regions (hsr).**

**Table 2 – EGFR immunohistochemistry and fluorescence in situ hybridisation comparison.**

| Stage | Markers | Variables | EGFR FISH-negative | EGFR FISH-positive | P[a] |
|---|---|---|---|---|---|
| I and II | EGFR IHC | | *n* = 22 | *n* = 23 | |
| | | Intense | 11 | 22 | 0.001 |
| | | Moderate | 10 | 1 | |
| | | Weak | 1 | 0 | |
| All | EGFR IHC | | *n* = 30 | *n* = 35 | |
| | | Intense | 15 | 30 | 0.004 |
| | | Moderate | 12 | 4 | |
| | | Weak | 3 | 1 | |

*Abbreviations*: IHC (immunohistochemistry) and FISH (fluorescence *in situ* hybridisation).
a $\chi^2$ MH test.

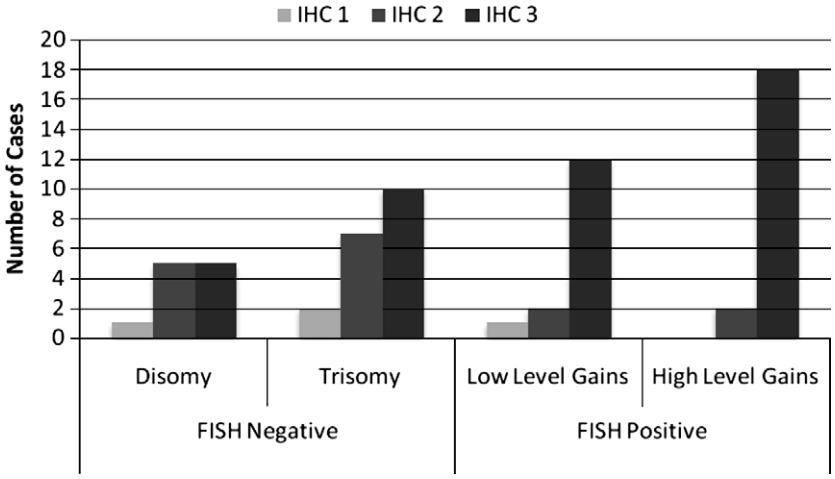


**Fig. 2 – Immunohistochemistry (IHC) results according to the different stratified fluorescence *in situ* hybridisation (FISH) groups with high EGFR protein expression corresponding with high gene copy number.**

**Table 3 – Radiotherapy response in Stage II OTSCC – comparison of EGFR immunohistochemistry and fluorescence in situ hybridisation.**

| Markers | Variables | pCR | Non-pCR | P[a] |
|---|---|---|---|---|
| EGFR IHC | | *n* = 7 | *n* = 24 | |
| | Intense | 4 | 17 | 0.216 |
| | Moderate | 3 | 4 | |
| | Weak | 0 | 0 | |
| EGFR FISH | | *n* = 5 | *n* = 19 | |
| | Positive | 3 | 14 | 0.558 |
| | Negative | 2 | 5 | |

*Abbreviations*: pCR (pathological complete responders); non-pCR (incomplete pathological remission); IHC (immunohistochemistry); and FISH (fluorescence *in situ* hybridisation).
a $\chi^2$ MH test.

### 3.3. *Survival outcome*

Patients with tumours showing an intense EGFR protein expression had a longer median survival time (22 months; range: 3–60 months) compared to patients with tumours expressing weak EGFR protein staining (14 months; range: 7–59 months). However, protein expression was not associated with overall patient survival in any individual stage or as a whole ($P = 0.494$). Median survival time for FISH-positive and FISH-negative groups were 21 (range: 3–59 months) and 28 (range: 4–60 months) months, respectively. EGFR gene status did not correlate with overall patient survival ($P = 0.683$). All 78 patients were included in the survival analyses.

**Table 4 – EGFR immunohistochemistry and fluorescence in situ hybridisation in relation to smoking.**

| Stage | Markers | Variables | Non-Smoking | Smoking | P[a] |
|---|---|---|---|---|---|
| All | EGFR IHC | | *n* = 24 | *n* = 40 | 0.166 |
| | | Intense | 20 | 25 | |
| | | Moderate | 3 | 14 | |
| | | Weak | 1 | 1 | |
| I and II | | | *n* = 20 | *n* = 29 | 0.009 |
| | | Intense | 19 | 18 | |
| | | Moderate | 1 | 11 | |
| | | Weak | 0 | 0 | |
| II | | | *n* = 13 | *n* = 17 | 0.043 |
| | | Intense | 12 | 10 | |
| | | Moderate | 1 | 7 | |
| | | Weak | 0 | 0 | |
| III and IV | | | *n* = 4 | *n* = 11 | 0.204 |
| | | Intense | 1 | 7 | |
| | | Moderate | 2 | 3 | |
| | | Weak | 1 | 1 | |
| All | EGFR FISH | | *n* = 19 | *n* = 34 | 0.008 |
| | | Positive | 15 | 14 | |
| | | Negative | 4 | 20 | |
| I and II | | | *n* = 15 | *n* = 23 | 0.001 |
| | | Positive | 13 | 7 | |
| | | Negative | 2 | 16 | |
| II | | | *n* = 12 | *n* = 14 | 0.024 |
| | | Positive | 11 | 7 | |
| | | Negative | 1 | 7 | |
| III and IV | | | *n* = 4 | *n* = 11 | 0.645 |
| | | Positive | 2 | 7 | |
| | | Negative | 2 | 4 | |

*Abbreviations*: IHC (immunohistochemistry) and FISH (fluorescence *in situ* hybridisation).
a $\chi^2$ MH test.

Overall survival and EGFR relationship was tested in all stages. Stages were tested individually and in groups. No association was detected between EGFR IHC or FISH to survival, as illustrated in Fig. 3.

## 4. Discussion

We investigated EGFR protein expression and gene copy number variation and its association with tumour progression, treatment prediction of preoperative radiotherapy and prognosis in OTSCC. We found that EGFR gene copy number significantly correlated with EGFR protein expression levels. Tumours that responded to radiotherapy showed less EGFR protein expression and the same trend was seen for EGFR gene copy number. Finally non-smokers had significantly higher EGFR protein expression and higher gene copy numbers in Stages I and II OTSCC.

EGFR protein expression levels and EGFR gene copy number were correlated in OTSCC. A majority of tumours with high EGFR gene copy number had intense EGFR protein expression in our study. The categories used for the evaluation for EGFR FISH were similar to those previously published.[10] Although genetic alterations (7p11 gain which harbours the EGFR gene) detected by array CGH as well as high EGFR protein expressions detected by IHC have been observed in oral cancer, a correlation between FISH and IHC results has never been shown.[11–13] Earlier studies on EGFR and head and neck cancers have mixed tissue types (anaplastic thyroid cancer, oesophageal cancer and laryngeal cancer).[14–16] Mrhalova and colleagues found no concurring evidence of correlation between EGFR gene copy number by FISH and protein expression using IHC in HNSCC.[14] Two studies found statistically significant correlation in head and neck tumours between FISH and IHC although Lee and colleagues did not observe any EGFR gene copy number increase.[15,16] When comparing Stage I versus more advanced tumours (Stages II, III and IV), a significance was observed with increasing EGFR FISH positivity ($P = 0.003$). Associations between EGFR overexpression and advanced tumour stage in oral cancer have been reported in the literature.[17,18]

EGFR protein expression by IHC has been suggested as a prognostic marker for head and neck cancer patients and has been shown to correlate with survival.[19,20] For oral cancer a correlation of EGFR with survival has been controversial with conflicting data.[16,21] Our results concurred with several studies which showed no association between EGFR protein expression and survival.[19,22] EGFR gene copy number by FISH as a prognostic factor has also rendered conflicting results, with our study showing no association.[16,19,23]

We found a trend between radiotherapy response and intense EGFR protein expression and high gene copy number in OTSCC. Previous studies have shown that high EGFR expression is found in tumour cells resistant to radiation-induced apotosis.[24] Studies have shown that cells exposed to

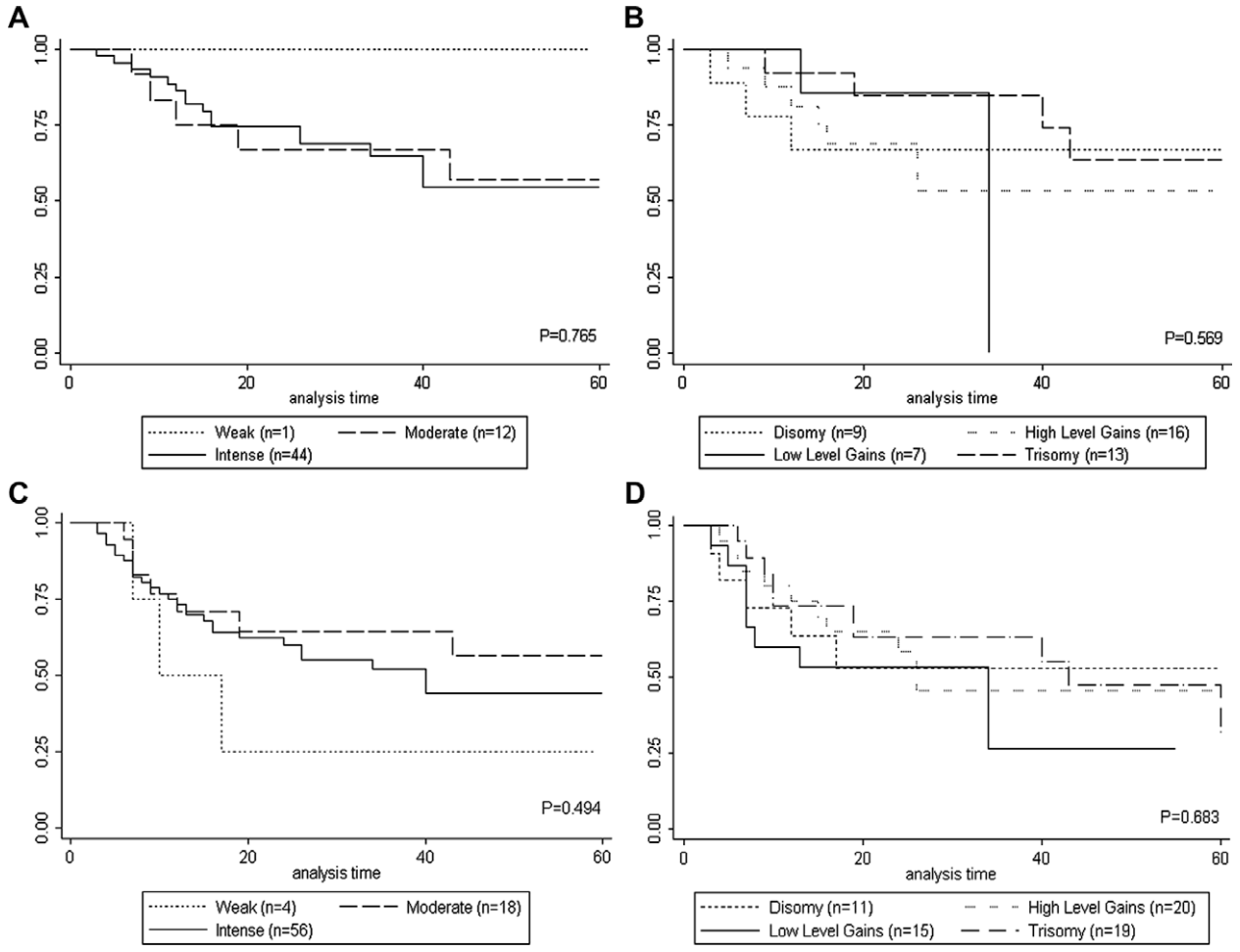


**Fig. 3 – Kaplan–Meier survival estimates in relation to EGFR immunohistochemistry (IHC) and fluorescence *in situ* hybridisation (FISH) expression variables: (A) EGFR IHC in Stages I and II OTSCC, (B) EGFR FISH in Stages I and II OTSCC, (C) EGFR IHC in all stages, and (D) EGFR FISH in all stages.**

EGF are protected from radiation damage (more radioresistant), which implies that EGFR may be regulating cellular response to radiation in order to protect it from radiation damage, whereas those pretreated with an EGFR inhibitor are radiosensitised.[25,26] Bonner and colleagues determined that EGFR inhibitor (cetuximab) improved locoregional control and reduced mortality without increasing common toxic effects associated with radiotherapy to the head and neck.[27] A high EGFR expression in the diagnostic pretreatment tumour biopsy may predict a worse radiotherapy effect and could possibly indicate patients who would benefit from adding an EGFR blocker to their therapy.

To our knowledge, this is the first study investigating EGFR gene copy number and protein expression levels exclusively in OTSCC. Most studies concerning the clinical value of markers for head and neck cancer mix tumours from all sub-sites, and show inconsistent results. We advocate the importance of investigating the sub-sites separately, because of proven differences among sub-sites. The presence of human papilloma virus (HPV), more common in the base of the tongue than in OTSCC, is associated with favourable prognosis in oropharyngeal cancer.[28]

In this study, non-smoking patients with OTSCC had higher EGFR gene copy number and protein expression levels than smoking patients with tumours. Smoking habits may thus be important when considering addition of EGFR inhibitor treatment. In previous studies, certain clinical parameters such as history of never smoking, Asian ethnicity, female gender and adenocarcinoma histology have shown EGFR inhibitor sensitivity in lung cancer.[29] Recent investigations on lung cancer have revealed that mutations in the TK domain of the EGFR gene are associated with EGFR inhibitor sensitivity making mutations a predictor for EGFR inhibitor treatment response.[30,31] According to a report by Pao and colleagues, non-smokers who were associated with EGFR inhibitor sensitivity in lung cancer also contained EGFR domain mutations in exons 19 and 21.[30] In addition, Miller and colleagues noted that non-smokers were more sensitive to gefitinib than smokers in lung cancer.[32] The presence of high EGFR expression in non-smokers may indicate the presence of EGFR mutations which could relate to EGFR inhibitor sensitivity. Recent reports have shown that EGFR mutations are rare in HNSCC although no reports have been made on OTSCC.[23] Future studies may therefore reveal the importance of using EGFR as a marker to determine patient response to the EGFR inhibitors in OTSCC.

Clinically useful molecular markers have become a topic of high priority due to the increasing cost of cancer treatment.

Techniques such as IHC and FISH are ways used to analyse biomarkers. However, IHC is a technique with subjective analyses, where reproducibility has always been an issue. After comparing IHC with FISH, we regard the latter to be a less subjective method and a method with better reproducibility when analysing the EGFR expression, but with a higher cost. Since our study showed that IHC and FISH results were in accordance, IHC may be the more cost-efficient method to use in clinical practice. A positive methodological aspect included simultaneous preparations, including: paraffin block cutting, staining of the EGFR antibody and analyses in all samples (see Section 2 for details). In addition, the time between paraffin block cutting and immunohistochemical staining did not exceed three weeks. One interpretation variance in our study may be due to the omission of a centromere-specific probe for chromosome 7, since the centromere probe of the same chromosome is typically used in studies on EGFR. Centromere 4 was used in our study to control for sectioning artefacts and was not used for interpretation of the EGFR results. Publications on OTSCC are few, therefore centromere 4 was chosen from head and neck cancer publications since it was the least affected chromosome with copy number changes.[33]

In summary, we found a good correlation between EGFR gene copy number by FISH and protein expression levels by IHC in OTSCC. Non-smoking tongue cancer patients exhibited higher EGFR gene copy numbers as well as protein overexpression than smokers. Finally, our data indicated that EGFR overexpression may help select patients who benefit from EGFR inhibitor therapy treatment.

## Conflict of interest statement

None declared.

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## REFERENCES

1. Shiboski CH, Schmidt BL, Jordan RC. Tongue and tonsil carcinoma: increasing trends in the US population ages 20–44 years. *Cancer* 2005;**103**:1843–9.
2. Mendelsohn J, Baselga J. Epidermal growth factor receptor targeting in cancer. *Semin Oncol* 2006;**33**:369–85.
3. Riesterer O, Milas L, Ang KK. Combining molecular therapeutics with radiotherapy for head and neck cancer. *J Surg Oncol* 2008;**97**:708–11.
4. Bentzen SM, Atasoy BM, Daley FM, et al. Epidermal growth factor receptor expression in pretreatment biopsies from head and neck squamous cell carcinoma as a predictive factor for a benefit from accelerated radiation therapy in a randomized controlled trial. *J Clin Oncol* 2005;**23**:5560–7.
5. Gupta AK, McKenna WG, Weber CN, et al. Local recurrence in head and neck cancer: relationship to radiation resistance and signal transduction. *Clin Cancer Res* 2002;**8**:885–92.
6. Huang SM, Harari PM. Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clin Cancer Res* 2000;**6**:2166–74.
7. Greene FL, Page DL, Fleming ID, et al. *AJCC cancer staging manual*. 6th ed. Springer; 2002.
8. Shah NG, Trivedi TI, Tankshali RA, et al. Molecular alterations in oral carcinogenesis: significant risk predictors in malignant transformation and tumor progression. *Int J Biol Mark* 2007;**22**:132–43.
9. Lim SC, Zhang S, Ishii G, et al. Predictive markers for late cervical metastasis in stage I and II invasive squamous cell carcinoma of the oral tongue. *Clin Cancer Res* 2004;**10**:166–72.
10. Hirsch FR, Varella-Garcia M, Bunn Jr PA, et al. Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. *J Clin Oncol* 2003;**21**:3798–807.
11. Garnis C, Campbell J, Zhang L, Rosin MP, Lam WL. OCGR array: an oral cancer genomic regional array for comparative genomic hybridization analysis. *Oral Oncol* 2004;**40**:511–9.
12. Bei R, Pompa G, Vitolo D, et al. Co-localization of multiple ErbB receptors in stratified epithelium of oral squamous cell carcinoma. *J Pathol* 2001;**195**:343–8.
13. Gebhart E, Ries J, Wiltfang J, Liehr T, Efferth T. Genomic gain of the epidermal growth factor receptor harboring band 7p12 is part of a complex pattern of genomic imbalances in oral squamous cell carcinomas. *Arch Med Res* 2004;**35**:385–94.
14. Mrhalova M, Plzak J, Betka J, Kodet R. Epidermal growth factor receptor – its expression and copy numbers of EGFR gene in patients with head and neck squamous cell carcinomas. *Neoplasma* 2005;**52**:338–43.
15. Lee DH, Lee GK, Kong SY, et al. Epidermal growth factor receptor status in anaplastic thyroid carcinoma. *J Clin Pathol* 2007;**60**:881–4.
16. Hanawa M, Suzuki S, Dobashi Y, et al. EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. *Int J Cancer* 2006;**118**:1173–80.
17. Massano J, Regateiro FS, Januario G, Ferreira A. Oral squamous cell carcinoma: review of prognostic and predictive factors. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006;**102**:67–76.
18. Bettendorf O, Piffko J, Bankfalvi A. Prognostic and predictive factors in oral squamous cell cancer: important tools for planning individual therapy? *Oral Oncol* 2004;**40**:110–9.
19. Sunpaweravong P, Sunpaweravong S, Puttawibul P, et al. Epidermal growth factor receptor and cyclin D1 are independently amplified and overexpressed in esophageal squamous cell carcinoma. *J Cancer Res Clin Oncol* 2005;**131**:111–9.
20. Brun E, Zatterstrom U, Kjellen E, et al. Prognostic value of histopathological response to radiotherapy and microvessel density in oral squamous cell carcinomas. *Acta Oncol* 2001;**40**:491–6.
21. Xia W, Lau YK, Zhang HZ, et al. Combination of EGFR, HER-2/neu, and HER-3 is a stronger predictor for the outcome of oral squamous cell carcinoma than any individual family members. *Clin Cancer Res* 1999;**5**:4164–74.
22. Sarbia M, Ott N, Puhringer-Oppermann F, Brucher BL. The predictive value of molecular markers (p53, EGFR, ATM, CHK2) in multimodally treated squamous cell carcinoma of the oesophagus. *Brit J Cancer* 2007;**97**:1404–8.
23. Chung CH, Ely K, McGavran L, et al. Increased epidermal growth factor receptor gene copy number is associated with

poor prognosis in head and neck squamous cell carcinomas. *J Clin Oncol* 2006;**24**:4170–6.
24. Panikkar RP, Astsaturov I, Langer CJ. The emerging role of cetuximab in head and neck cancer: a 2007 perspective. *Cancer Invest* 2008;**26**:96–103.
25. Akimoto T, Hunter NR, Buchmiller L, Mason K, Ang KK, Milas L. Inverse relationship between epidermal growth factor receptor expression and radiocurability of murine carcinomas. *Clin Cancer Res* 1999;**5**:2884–90.
26. Wollman R, Yahalom J, Maxy R, Pinto J, Fuks Z. Effect of epidermal growth factor on the growth and radiation sensitivity of human breast cancer cells in vitro. *Int J Radiat Oncol Biol Phys* 1994;**30**:91–8.
27. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *New Engl J Med* 2006;**354**:567–78.
28. Lindquist D, Romanitan M, Hammarstedt L, et al. Human Papillomavirus is a favourable prognostic factor in tonsillar cancer and its oncogenic role is supported by the expression of E6 and E7. *Mol Oncol* 2007;**87**:1–6.
29. Sequist LV, Lynch TJ. EGFR tyrosine kinase inhibitors in lung cancer: an evolving story. *Ann Rev Med* 2008;**59**:429–42.
30. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* 2004;**101**:13306–11.
31. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *New Engl J Med* 2004;**350**:2129–39.
32. Miller VA, Kris MG, Shah N, et al. Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. *J Clin Oncol* 2004;**22**:1103–9.
33. Squire JA, Bayani J, Luk C, et al. Molecular cytogenetic analysis of head and neck squamous cell carcinoma: by comparative genomic hybridization, spectral karyotyping, and expression array analysis. *Head Neck* 2002;**24**:874–87.